EXHIBIT A

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ADJUVANT EFFECTS OF SEMI-SYNTHETIC CHOLESTEROL DERIVATIVES.

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INTRODUCTION

The cationic cholesterol derivative, 3 β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), is a cationic amphiphile composed of a cholesterol moiety which is chemically linked via a 3-carbon spacer to a cationic dimethylamino head group (Figure 1). This derivative was designed and used to promote or facilitate the transfer of nucleic acids into cells (i.e. transfection) (1).

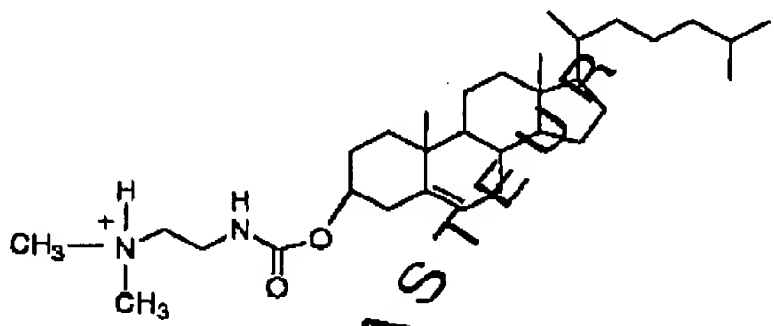

Figure 1: Stuture of DC-Chol

We believe that DC-Chol and more generally the cationic lipids described in US patent number 5,283,185 - to the University of Tennessee USA and McMaster University, Ontario, Canada - are potentially excellent candidates for vaccine adjuvantation.
These compounds comprise a lipophilic group derived from cholesterol, a linker bond, a spacer arm including from about 1 to 20 Carbon atoms in a branched or unbranched linear alkyl chain and a cationic amino group.

These cholesterol based amphiphiles are easely synthesized by condensation of a reactive cholesterol derivative (e.g. Cholesteryl hemisuccinate, cholesteryl chloroformate) with a hydrophilic head group. These compounds are metabolizable; they can be synthesized with hydrolysable linker bonds (e.g. ester, amide, carbamoyl) which are degraded in cells. In addition the cationic cholesterol derivatives have a reduced cytotoxicity because they do not inhibit cellular Protein Kinase C.

It is of particular interest that the cholesterol based amphiphiles form particles (e.g. liposomes, micelles) when dispersed in water or an aqueous buffer. Such particles can be used to entrap and deliver antigenic peptides or proteins in the body.

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We believe that particles made of the cationic amphiphile DC-Chol (or analog) which are used for transfection and display fusogenic properties could be used to deliver antigens in the cytoplasm of cells. For instance, a commercially available transfection reagent, the cationic lipid (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimlethylammonium methyl sulfate (DOTAP), has been used to direct the Herpes Simplex Virus glycoprotein B into the class I major histocompatibility complex antigen-presentation pathway and to induce a specific CTL response against herpes infected cells (2).

In the present report we show that particles made of an amphiphile with intrinsic adjuvant and fusogenic properties (e.g. DC-Chol) are particularly suitable for the adjuvantation and delivery of antigenic peptides or proteins in vaccination protocols.

RESULTS AND DISCUSSION

Synthesis of DC-Chol.

3 β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was synthesized according to Gao and Huang (1). In brief, Cholesteryl chloroformate (Sigma) was reacted with a 4 fold molar excess of N,N-dimethylethylendiamine (Sigma) in dry chloroform at 0°C. DC-Chol was purified by recristallisation in absolute ethanol and obtained with a 38% yield. Analytical data (Rf, NMR and mass spectrometry data) were in agreement with published data.

Dispersion of DC-Chol in water and association with monovalent flu vaccine.

DC-Chol vesicles were obtained by rapid injection of an ethanolic solution of the amphiphile in distilled water (3). One hundred micrograms of DC-Chol were dissolved in 100 µl of ethanol. Seventy five microliters were injected through a Hamilton syringe into 3 ml of water kept under stirring at 45°C. After 5 minutes of additional stirring at 45°C, the vesicles were placed at room temperature and mixed with 200 µl of monovalent flu vaccine (PMsv split vaccine, strain A/Singapore/6/86, N° MIA SJ03, 250 µg HA/ml) and dialyzed overnight against PBS at 4°C.

By using radioiodinated HA as a tracer, we found that 50-90% of HA associates with the membrane bilayers upon the simple mixing of flu vaccine with preformed liposomes in the above described conditions. The amount of liposome-bound HA was measured after purification of the liposomes by floatation through a gradient of metrizamide and was depending on the lipid composition of the vesicles. Neutral liposomes bound up to 50% of HA and cationic liposomes bound up to 90% of HA.

The lipid injection method yields small unilamellar liposomes with a diameter of about 50 nm (3). Vesicles with similar caracteristics can be obtained by other techniques (e.g. rehydration of dried lipids and sonication or extrusion). The injection technique is preferred because it allows reproducible and large scale manufacturing.

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Immunization of BALB/c mice with flu vaccine associated with DC-Chol liposomes.
BALB/c mice (4 mice per group) were immunized by subcutaneous injection of monovalent flu vaccine (5 µg HA) alone or associated with DC-Chol liposomes (2.3 mg of DC-Chol). Three weeks after the injection, the level of IgG antibodies in the sera of immunized mice was measured by an ELISA assay with the vaccine adsorbed to microtiter plates. After incubation of the plates with the sera of immunized mice, the level of IgG antibodies was indicated with an anti-mouse IgG-horseradish peroxydase conjugate. The association of the flu vaccine with DC-Chol liposomes produced a 40 fold increase in the specific IgG response. The specific IgG titers in the sera of immunized mice were about 30,000 and 1,200,000 in mice receiving respectively the vaccine alone (5 µg HA) and the vaccine associated with DC-Chol liposomes (5 µg HA; 2.3 mg DC-Chol). Titers in neutralizing antibodies are currently being determined.

The formulation of flu vaccine with DC-Chol liposomes induced high antibody titers even after a storage period of one month at 4°C. However this cationic liposome formulation has not been optimized yet in terms of stability and doses.
Since phosphate ions can interact with cationic liposomes, the stability of the formulation may improve by suppressing the dialysis step after mixing the vaccine with DC-Chol liposomes or by replacing PBS with saline in this dialysis step.
The dose of 2.3 mg of DC-Chol liposomes for 5 µg of HA was chosen arbitrary. We consider this dose as the upper limit of the dose range in mice. A dose/response study aiming to define the lower limit of the dose range is currently in progress. We believe that the amount of DC-Chol can be lowered or partially replaced with a co-lipid. The co-lipid can be a neutral or acidic phospholipid which may be preferentially selected from the group consisting of phosphatidylcholine and phosphatidylethanolamine.

We believe also that the association of flu vaccine with DC-Chol will direct HA and NP into the class I major histocompatibility complex antigen-presentation pathway and induce a specific CTL response against influenza infected cells. The specific CTL response induced in mice immunized with flu vaccine associated with DC-Chol liposomes is currently under investigation.

CONCLUSION

In conclusion, we have shown that the cationic amphiphile DC-Chol is a powerfull immunoadjuvant. The association of the flu vaccine with DC-Chol liposomes produces a 40 fold increase in the specific anti-influenza IgG response in BALB/c mice. From results obtained by others with viral antigens associated with fusogenic cationic lipids [e.g. DOTAP (2)], we expect the DC-Chol formulation to induce specific CTL responses in vaccination protocoles.

We extend the considerations made on DC-Chol to the cationic lipids described in US patent number 5,283,185 and more generally to any cationic amphiphile comprising a lipophilic moiety derived from a sterol (e.g.

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cholesterol, phytosterol, ergosterol) and a cationic headgroup or a mono or polysaccharide.

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(3) Kremer J.M.H., Esker M.W.J.v.d., Pathmamanoharan C., and Wiersema P.H., Vesicles of variable diameter prepared by a modified injection method. Biochemistry, 16, 3932-3935 (1977).

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